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Diet fatty acid profile, membrane composition and lifespan: an experimental study using the blowfly (Calliphora stygia)

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Keywords

Maximum lifespan, membrane fatty acids, diet fatty acids, Polyunsaturates, membrane pacemaker

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Diet fatty acid profile, membrane composition and lifespan: an experimental study using the blowfly (*Calliphora stygia*).

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Abstract

The membrane pacemaker theory of ageing proposes that the polyunsaturated fatty acid (PUFA) composition of membrane lipids of a species is an important determinant of its maximum lifespan. We report three experiments using the blowfly *Calliphora stygia*, where this theory was tested by manipulation of dietary fat profile. Although the fat profile of the larval diet resulted in small alterations of individual membrane fatty acids, it had no effect on the peroxidation index (PI) of membrane lipids and furthermore had no effect on maximum lifespan. Similarly, manipulation of the fat profile of the adult diet resulted in small changes in individual fatty acids, but had no effect on the PI of membrane lipids. There was a small increase in maximum lifespan when adult diet was supplemented with PUFA in form of vegetable oils but no effect when diet was supplemented with pure PUFA. This difference is possibly due to antioxidant content of vegetable oils. The relative refractoriness of membrane PI to dramatic changes in response to diet is similar to the situation in the rat. These results also indicate the blowfly is unable to convert 18-carbon PUFA to more highly polyunsaturated 20- and 22-carbon PUFA.

(194 words)

1. Introduction

Animal species have distinctive maximum lifespans and there are several theories about the mechanisms of ageing that might determine lifespan, but no universal agreement about these processes. One theory, the 'membrane pacemaker' theory, is a modification of the oxidative stress theory of ageing and proposes that the fatty acid composition of membranes (especially mitochondrial membranes) may be an important component determining maximum lifespan and ageing of animals (Pamplona et al., 1998; Hulbert, 2005). This theory is based on the fact that fatty acids differ dramatically in their susceptibility to peroxidation (Holman, 1954). Fatty acids include saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), but only PUFA undergo significant lipid peroxidation, with the more polyunsaturated the PUFA the more prone it is to peroxidative damage. By contrast, the SFA and MUFA are highly resistant to peroxidative damage. The membrane pacemaker theory proposes that the link between membrane composition and lifespan determination is that the peroxidative susceptibility of membranes determines the degree of lipid peroxidation and the consequent damage to other important biomolecules from the products of PUFA peroxidation (see Hulbert et al., 2007).

The development of the 'membrane pacemaker' theory of ageing originated largely from correlations between membrane fatty acid composition and maximum lifespan of mammal and bird species (for review see Hulbert et al., 2007). More recent studies also provide evidence of correlations between membrane composition and longevity in some invertebrate species, including honeybees (Haddad et al., 2007), bivalve molluscs (Munro and Blier, 2012) and the nematode *Caenorhabditis elegans* (Shmookler Reis et al., 2011). Most evidence to date is correlational and therefore requires manipulation of membrane fatty acid composition to determine if the relationship is one of cause and effect. An obvious experimental test of the 'membrane pacemaker' theory would be to alter membrane fatty acid composition by diet manipulation, and determine whether such membrane changes also alter animal longevity in a predicted manner.

Because of both their relatively short adult lifespans and low husbandry costs, some invertebrate species (e.g. *Drosophila* and *C. elegans*) have been used to experimentally test theories of ageing. We have previously examined ageing in the blowfly, *Calliphora stygia* and suggested it is a good model organism to study mechanisms of ageing as it has both a short adult lifespan and the additional advantage of being large enough (body mass ~70 mg) to measure the biochemistry and physiology of individuals (Hulbert et al., 2004; Ujvari et al., 2009; Kelly et al., 2013). In blowflies, the larval life stage is the one with the greatest growth and thus involves considerable membrane formation while the adult life stage involves negligible growth (and thus negligible new membrane formation) and changes in membrane composition will likely only be the result of membrane lipid turnover. We have experimentally investigated both life stages and report the findings of three separate experiments designed to test the 'membrane pacemaker' theory of ageing. Testing whether manipulation of dietary fatty acid composition changes membrane fatty acid composition and consequently also changes adult lifespan of this blowfly. The first experiment changed membrane fatty acid composition of blowfly larvae (by manipulation of the larval diet) and determined whether lifespan of the adult blowfly was affected. The other two experiments manipulated fatty acid composition the adult diet of the blowfly and whether these treatments affected adult lifespan of the blowfly.

2. Materials and Methods

2.1. Experiment #1: the effect of larval diet on membrane composition and adult longevity.

In nature, eastern golden-haired blowflies (*C. stygia*) are carrion feeders, and in the laboratory larvae can be raised using liver. A large number of adult *C. stygia* were kept in breeding cages (35 cm x 35 cm x 25 cm) with unlimited access to water and sheep liver. Females deposited eggs on the surface of the liver, which were gently collected with a small paintbrush and then pooled before being randomly transferred to petri dishes containing one of six different types of larval food/substrate (replicate dishes for each diet and ~15 eggs per petri dish). Each petri dish was

then placed in a container with an insect screen lid. After larvae emerged they were provided with regular replacement of the specific fresh food in new petri dishes and allowed to progress to pupation.

One larval food consisted of 50% agar (Technical No 3 grade, Oxoid Ltd, Basingstoke, England) and 50% homogenised sheep liver, while the other five foods consisted of 50% agar : 35% homogenised liver : 15% oil. Five different oils were chosen for these diets to emphasise different classes of fatty acids, namely SFA, MUFA, n-6 PUFA and n-3 PUFA (Fig. 1). The n-3 PUFA examined were of two types; shorter 18-carbon n-3 PUFA (plant based) and the longer 20- and 22-carbon n-3 PUFA (fish based). The specific oils used were; (i) beef tallow (Allowrie Prime Beef Dripping, Rowville, Vic, Australia), (ii) olive oil (Lupi cold pressed extra virgin, Gosford, NSW, Australia), (iii) sunflower oil (Crisco, Goodman Fielder, Macquarie Park, NSW, Australia), (iv) flaxseed oil (Healthy Life cold-pressed virgin, Milperra, NSW, Australia), and (v) fish oil (Melrose Omega 18/12 Fish Oil, Mitcham, Vic, Australia).

Prior to eclosion, pupae were transferred to individual 125 cm^3 plastic containers (44 mm diameter x 90 mm long) with foam stoppers. Each had a small plastic food dish and blowflies were provided daily with fresh 0.2 M sucrose solution in excess to their requirements (Hulbert et al., 2004). Blowflies were individually weighed (to ±0.1 mg) on a Mettler Toledo AB204-5 balance at one and ten days post-eclosion. Six larvae (3^{rd} instar, after 7-10 days on larval food) and six freshly pupated adults were randomly sampled from each treatment and immediately frozen in liquid nitrogen for later analysis of the fatty acid composition of their phospholipids (i.e. membrane lipids). All other individuals were used for lifespan determination, with time between eclosion and death determined from daily censuses. This permitted calculation of average and maximum lifespan for each treatment population, maximum lifespan being defined as the mean lifespan of the longest-living 10% of the population. Three fresh samples of each food were also frozen in liquid nitrogen for later analysis of the fatty acid composition of food lipids.

Because sample size for lifespan determination averaged only 22 blowflies per treatment in the first run of this experiment (#1a), it was repeated (#1b) using a larger number of blowflies per treatment (average sample size of 97 blowflies per treatment), but comparing only three of the larval foods (those containing either olive oil, sunflower oil or fish oil), with lifespan and body mass recorded. No samples were analysed for fatty acid composition from this second run of experiment 1.

2.2. Experiment #2: the effect of adult SY diet with added vegetable oils on membrane composition and longevity.

Pupae of *C. stygia,* were purchased from a commercial breeder (Sheldon's Bait, Parawa, SA, Australia) to examine the effect of 4 different low-fat sugar-yeast-agar (SY) diets on adult longevity as described in Ujvari et al. (2009). Prior to eclosion, pupae were randomly divided among eight rearing cages, representing duplicate cages for each of the four diets. Cages were made by covering an aluminium frame (height 35 cm x width 40 cm x depth 35 cm = 49,000 cm^3) with mosquito mesh. An area on the front of each cage was covered with pantihose (with the ends cut off each leg) allowing daily removal of dead flies, occasional sampling of live flies, and daily replacement of food and water dishes, without escape of live flies. Following mass eclosion of blowflies in a given cage, the remaining non-eclosed pupae were removed and this was declared day 0 for all adult flies in that cage. For all experiments, fly cages were housed in a temperature- and humidity-controlled room on a 12 hour day: 12 hour night photoperiod. Temperature and humidity were continuously monitored using a Gemini Tinyview Datalogger (Chichester, UK) and averaged 28 $\pm 1^{o}$C and 80 $\pm$5% R.H. (mean $\pm$ SD) during the experiment.

Blowflies were provided with *ad libitum* access, throughout their adult life, to Petri dishes containing one of four diets that differed only in fat profile. The diets were identified as either: (i) SY + SFA, (ii) SY + MUFA, (iii) SY + n-6 PUFA, or (iv) SY + n-3 PUFA and consisted of a standard SY diet with 3 g added vegetable oil per 100 ml diet. The basic composition of the SY diet

was 50 g Enoferm M1 general wine yeast (Laffort Oenologie), 50 g sugar, 0.125 g nipagen (Sigma-Aldrich), 1.8 g agar technical No. 3 (Oxoid) and 100 ml water, the fatty acid composition of this standard SY diet is presented in Table 1. The water was heated to 100^{o}C and, with continuous stirring, the agar, yeast, sugar and nipagen gradually dissolved with 3 g of a specific vegetable oil also added to this mixture. The following vegetable oils were used: coconut oil (Aclara Health Organic Gourmet Cold Pressed Extra Virgin, Mount Crosby, QLD, Australia) for SY +SFA diet; olive oil (Lupi Cold Pressed Extra virgin, Gosford, NSW, Australia) for SY +MUFA diet; safflower oil (Healthy Life Cold Pressed, Gosford, NSW, Australia) for the SY +n-6 PUFA diet; flaxseed oil (Nutralife Hi-Omega, Moorebank, NSW, Australia) for the SY +n-3 PUFA diet. This food solution was evenly distributed in Petri dishes and stored in closed containers at 4^{o}C after solidification.

The population sizes ranged between 222 and 292 flies per cage. Dead flies were removed daily and some live blowflies were also removed at days 0, 21 and 42, immediately frozen at -80^{o}C, and then analysed later for fatty acid composition (see Table 2 for number of flies). These blowflies were not part of lifespan determination and in all cases equal numbers of live male and female blowflies were sampled for fatty acid analysis. Because there were no significant differences in average lifespan between the duplicate cages, data from them were combined (i.e. treated as a single cage) for lifespan calculations. Average lifespan and maximum lifespan (mean of longest-living 10% of blowflies) were determined for each treatment population.

2.3. Experiment #3: the effect of adult SY diet with added individual fatty acids on membrane composition and longevity.

In order to better understand the relationship between dietary fat profile and membrane composition and longevity, we followed the same protocol as experiment #2, except that instead of vegetable oils (which each contain a distinctive mixture of fatty acids as well as endogenous antioxidants), individual 18-carbon fatty acid methyl esters were added to the standard SY diet. Pure methyl esters of 18:0, 18:1n-9, 18:2n-6 and 18:3n-3 were obtained from Sigma-Aldrich

(Sigma-Aldrich, Sydney, NSW, Australia). No antioxidants were added to the food mixtures, however food was stored frozen and only removed to briefly defrost before being placed into the fly cages. Five populations (~120 flies per cage, no replicate cages) were fed different diets for their entire population lifetime. One population was fed the standard SY diet, while the other four populations were fed the standard SY diet with a specific fatty acid methyl ester added (3 g per 100 ml of food).

Blowflies had *ad libitum* access to water and to Petri dishes containing the specified food. Food dishes were changed daily, and food consumption was determined by weighing food dishes before and after their time in the cage using a Mettler Toledo AB204-5 balance (to $\pm$0.1 mg). Food consumption values were corrected for mass loss by evaporation as determined from food dishes placed outside the cages in the same room.

Live flies (n = 12) were removed from cages at days 0, 7, 14, 21, 28 and 42 and frozen at -80°C for later phospholipid fatty acid analysis of whole flies and isolated thoracic mitochondria. The remaining flies were used for longevity measures, with any live flies removed from cages not included in the lifespan calculations. For each population, average lifespan and maximum lifespan (= mean of individual longevities of longest-living 10% of population) were determined.

2.4. Isolation of blowfly thoracic mitochondria.

The thorax was separated from individual frozen blowflies from experiment 3 and homogenised in 10 ml of ice-cold homogenisation medium using glass-glass homogenisers. The homogenisation medium was 100 mM KCl, 25 mM Tris-HCl, 2 mM EGTA (ethylene glycol tetraacetic acid) adjusted to pH = 7.4 @ 4^{o}C with the protease subtilisin A type VIII (Sigma-Aldrich #P5380) added at a concentration of 1 mg / 10 ml. Homogenates were kept on ice for at least 10 min, after which they were transferred to centrifuge tubes and centrifuged for 10 min @ 1000 g at 4^{o}C. The supernatant was then centrifuged for a further 10 min @ 10,000 g at 4^{o}C and lipids were extracted from the resulting pellet for fatty acid analysis. This pellet is assumed to represent

thoracic (i.e. predominantly flight muscle) mitochondria as previous experiments using this precise protocol on thorax from live flies produce a pellet that consumes oxygen when provided substrate and responds appropriately to mitochondrial inhibitors (M. Montgomery and A.J. Hulbert, unpublished observations).

2.5. Measurement of fatty acid composition.

Depending on the particular experiment, total lipids were extracted from either (i) larval blowflies, (ii) adult blowflies, (iii) mitochondrial preparations, or (iv) food samples in glass-glass-homogenisers with ~20 volumes of chloroform:methanol (2:1 v/v) containing butylated hydroxytoluene (0.01% w/v) as an antioxidant. Phospholipids were separated from total lipids of larvae, adult blowfly and mitochondrial samples (but not food samples for which total lipids were analysed) and fatty acid composition of these lipids determined by methods previously described (Haddad et al., 2007). We will identify individual fatty acids using a number system where the first number represents the chain length, the second number is the number of double bonds and the third is the position of the most terminal double bond (e.g. linoleic acid is identified as 18:2 n-6). Based on these fatty acid determinations, we have calculated a Peroxidation Index (PI), which is a parameter that indicates the calculated relative susceptibility of particular lipid sample to peroxidation. It is calculated using the formula PI = (0.025 x % monoenoics) + (1 x % dienoics) + (2 x % trienoics) + (4 x % tetraenoics) + (6 x % pentaenoics) + (8 x % hexaenoics) (see Hulbert et al., 2007).

2.6. Statistical analyses.

Data are presented as means ± the standard error of the mean (S.E.M.). All statistical analyses were performed using GraphPad Prism Version 5 (GraphPad Software, San Diego, CA, USA) and JMP 9.0.2 (SAS Institute, Cary, NC, USA). The difference between means was determined by T-test or ANOVA (with Tukeys HSD post-hoc test). Where data was found to fail

normality or equal variance analyses, Mann-Whitney t-test or Kruskal-Wallis ANOVA (with Dunns post-hoc test) were used. Statistical significance between means was accepted at $P < 0.05$, for analyses using multiple comparisons significance was set at $P < 0.01$.

3. Results

3.1 Effect of larval diet on membrane fatty acid composition and adult longevity of blowflies.

The relative composition of fatty acids (% total fatty acids) of (i) total lipids of six different larval foods, (ii) phospholipids (=membrane lipids) isolated from the 3rd instar pupae raised on these foods, and (iii) phospholipids isolated from the thorax of newly-eclosed adults from the different larval populations are presented in pie-chart format in Figure 1 (see Supplementary Table 1 for values and statistical differences). The values for the adults are overwhelmingly dominated by flight muscle composition as validated in a separate series of measurements that showed no difference between whole thorax and isolated flight muscle (Usher and Hulbert, unpublished results).

As can be seen from Figure 1, although there were large differences in the fatty acid composition between the different foods, there were much more limited differences between fatty acid composition of phospholipids from larvae fed the different diets, and from adults derived from these larvae. For example, while the SFA content of the diets varied from 13% of total fatty acids (flaxseed oil diet) to 52% (tallow diet), there was negligible difference in membrane SFA between the larvae (SFA ranged from 27% to 31%) and also between the adults (SFA ranged from 17% to 21%). Thus, it appears that the SFA content of membrane lipids is relatively homeostatically regulated with respect to diet variation. For MUFA, n-6 PUFA and n-3 PUFA, there was variation among larvae and adults but, similarly, it was not as great as the variation between the diets. The MUFA content of food ranged from 21% to 73%, while the range for phospholipids from larvae was 27% to 54% and from adults it was 19% to 55%. The n-6 PUFA content of food ranged from 3% to 56%, while membrane n-6 PUFA ranged from 8% to 41% in larvae, and from 15% to 56% in

adults. For n-3 PUFA content the diets ranged from 1% to 50% while for larval phospholipids ranged from 4% to 30% and from 5% to 36% for adults. The fatty acid composition of blowfly phospholipids was most responsive to dietary n-6 PUFA content, less responsive to the diet n-3 PUFA and diet MUFA content and almost unresponsive to diet SFA content (Fig. 1).

Because a fatty acid is present in the diet it does not mean that it will be a component of membrane lipids. For example, the most polyunsaturated fatty acid (22:6 n-3) was a significant component of the fish oil diet, and was present in smaller amounts in all other diets, however it was absent from membrane lipids among all treatment groups, presumably excluded by some biochemical mechanism regulating membrane composition (see Figure 1).

The relationship between diet PI and membrane lipid PI is plotted in Figure 2. As can be seen from this figure, although there was considerable variation in PI of the different diets (ranging from 14 to 222), there was relatively little diet-induced variation in PI of membrane lipids in both larvae (ranging from 53 to 83) and adult thoraces (ranging from 83 to 105). In this figure is also plotted the line of conformity, which indicates a slope of 1.0 if membrane lipid PI perfectly conformed to diet PI. The slope of this relationship for phospholipids (= membrane lipids) of larvae is 0.14 while that for adult thorax phospholipids it is 0.05 and neither is significantly different from a zero slope. Thus, in the blowfly, despite extensive variation in diet, the peroxidative susceptibility of blowfly membranes appears to homeostatically regulated at a relatively constant level. This indicates that although diet increased the relative abundance of some individual fatty acids in membrane lipids (Fig. 1), that there were reduced abundance of other fatty acids that were compensatory, at least with respect to PI value.

It should be noted that although the fatty acid composition of larval and adult phospholipids varied far less than the fatty acid composition of the different larval foods, there were consistent larval-adult differences irrespective of diet. These were that adult phospholipids had less SFA and more n-6 PUFA and more n-3 PUFA than the respective larval phospholipids. It is possible these differences reflect tissue differences rather than developmental changes because adult

phospholipids were only derived from thorax (and predominantly flight muscle) while larval phospholipids were from the whole organism.

The average adult lifespan and maximum adult lifespan of blowflies fed different diets as larvae but the same diet as adults (i.e. sugar and water) are graphically presented in Figure 3 (survival curves are presented in Supplementary Figure 1). As can be seen from this figure, both experiments #1a and #1b show no effect of diet on adult lifespan. There were no significant differences in average longevity ($P = 0.185$ for experiment #1a, $P = 0.127$ for experiment #1b) or maximum longevity ($P = 0.091$ for experiment #1a and $P = 0.093$ for experiment #1b) of adult blowflies fed different diets as larvae. Neither average lifespan nor maximum lifespan was influenced by larval diet and any consequent changes this had on membrane composition of adults.

3.2 Effect of adult diet with added vegetable oils on membrane fatty acid composition and longevity of blowflies.

The membrane (=phospholipid) fatty acid composition of whole blowflies from these populations is presented in Table 2. Whereas in experiment #1 membrane fatty acid composition was measured only at the beginning of adult life (i.e. day 0 adults), in this experiment (#2) it was measured at day 0 and day 21 (~30% of maximum lifespan) and day 42 (~60% of maximum lifespan) to determine how the different adult diets changed membrane composition of adults. For all four treatments, the greatest change in membrane composition was apparent between the day 0 and day 21 measurements, which exceeded the effect of diet treatment. In all populations there was a substantial decrease in SFA content between newly eclosed blowflies, and those measured at 21 days (even in those blowflies fed the SFA-supplemented adult diet). However there was no difference in SFA content between days 21 and 42 for any of the diet treatments. An increase in MUFA content of membrane lipids was also seen in 21-day old blowflies compared to those newly eclosed, predominantly due to increased 16:1n-7 content with negligible change in 18:1n-9 content. This MUFA change was influenced by diet but apparently in an indirect manner, in that only

blowflies fed SFA-enriched diets showed a significant increase in 16:1n-7 content. Furthermore, the dominant fatty acid in the MUFA-enriched diet is 18:1n-9, however there was no significant difference in the 18:1n-9 content of membrane lipids of blowflies fed this diet compared to the other diets. Enrichment of the diet with both n-6 PUFA and n-3 PUFA resulted in an increase in the relative abundance of the dominant PUFA in each diet (18:2n-6 and 18:3n-3 respectively) but, interestingly, not in the 20-carbon PUFA (i.e. 20:4n-6 and 20:5n-6) that are made from these 18-carbon fatty acids. Indeed, the decrease (~3-fold over 42 days) in both of these highly polyunsaturated 20-carbon fatty acids was the greatest relative change in membrane fatty acid composition measured for all diets. This decrease was largely responsible for the age-related decrease in PI of membrane lipids, which was seen in all populations with the exception of blowflies fed n-3 PUFA enriched diets. The decrease in 20-carbon PUFA from day 0 to day 42 was almost identical in all diet groups and can be calculated to represent 44, 40, 43 and 44 units of PI respectively for the SFA, MUFA, n-6 PUFA and n-3 PUFA treatments.

Vegetable oil-supplemented diets fed to *C. stygia* during their entire adult lives had some significant effects on longevity (Fig 4; see Supplementary Figure 2 for survival curves). There were no significant longevity differences between the populations fed diets enriched with SFA and MUFA and these had the shortest lifespans. Those flies fed diets enriched in both types of PUFA had significantly longer average and maximum lifespans than the SFA-enriched and MUFA-enriched diets while the maximum lifespan of blowflies provided with extra n-3 PUFA had a significantly longer maximum lifespan ($P < 0.0001$) than those fed the n-6 PUFA diet .

3.3. Effect of SY diet supplemented with 18-carbon fatty acids on longevity and membrane fatty acid composition.

There were a number of rationales for the design of this experiment (#3). In both experiments #1 and #2, fatty acids were presented in the diet in the form of triglycerides and although the oils were chosen because each one contained predominantly a particular type of fatty

acid, all these oils, in fact, contained a mixture of several fatty acids as well as some non-fatty acid molecules. In view of our relative inability in experiments #1 and #2 to substantially change membrane fatty acid composition in blowflies, and in order to bypass the processes involved in the digestion of triglycerides, experiment #3 was designed to examine whether the provision of dietary fatty acids as individual methyl esters was capable of significantly altering membrane composition. A second rationale was that, although it is common practice in dietary experiments to use different oil mixtures, these oils are not pure triglycerides but almost always contain significant amounts of fat-soluble antioxidants, the levels and types of which differ between different types of oils. This incidental provision of antioxidants might have unforeseen consequences in experiments examining diet effects on longevity. For example, any detrimental effects of fatty acids in a specific oil may be counterbalanced by beneficial effects of antioxidants in that oil. The provision of pure fatty acids (as methyl esters) in the diet without these additional oil-based antioxidants avoids this potential problem. Experiment #3 was limited to examination of the effects of enrichment of the standard SY diet with specific 18-carbon fatty acids (namely 18:0, 18:1n-9, 18:2n-6, 18:3n-3) as well as a control 'no-added-fat' SY diet, with the restriction to 18-carbon fatty acids mostly as a result of the expense of single methyl esters as a lifetime food source for populations of blowflies. The results from experiment #2 indicated that there were age-related changes in phospholipid fatty acid composition during the adult life of blowflies (i.e. comparison of days 0, 21 and 42) that was relatively unaffected by diet and thus to more fully document this change, a greater number of ages were analyzed in experiment #3. A fourth rationale for the design of experiment #3 was to examine if the changes measured in whole blowfly phospholipids (i.e. pooled membrane lipids from the whole organism) in experiments #1 and #2 were also manifest in a more pure specific membrane important for ageing, the mitochondrial membrane. We have determined that thorax phospholipids are predominantly derived from flight muscle (Usher and Hulbert, unpublished results) and have previously isolated functional mitochondria from thoraces of individual blowflies (Montgomery and Hulbert, unpublished results). Thus in experiment 3 the time course of the effect of individual diet

fatty acids on fatty acid composition of whole blowfly phospholipids and thorax mitochondrial phospholipids was compared.

The diet-induced changes in the major fatty acids of both whole blowfly and mitochondrial phospholipids are presented in Figure 5. As can be seen from this figure, the changes were the same whether measured in mitochondrial phospholipids or whole blowfly phospholipids. Although the age-related changes in membrane fatty acid composition during adult life were similar to those observed in the previous two experiments, there were some differences. In the blowflies fed the standard SY diet (i.e. with no-added-fat), there was a decrease in 16:0 but no change in 18:0, a substantial increase in 16:1n-7 content but no change in 18:1n-9 content. With respect to both types of PUFA, there were substantial and continual decreases in both 20:4n-6 and 20:5n-3 content of membrane lipids with very small decreases in 18:2n-6 and 18:3n-3 content. The addition of large amounts of 18:0 to the adult diet resulted in no significant change to membrane fatty acid composition nor the age-related changes. The addition of large amounts of 18:1n-9 to the adult diet resulted in a significantly greater amount of 18:1n-9 in membrane lipids compared to blowflies fed the other diets. However, there was little change in the 18:1n-9 content of membrane lipids throughout the adult life of the blowfly (from ~32% to ~30%) despite the very large amounts provided in the diet. This was in contrast to the substantial increase in 16:1n-7 content of membrane lipids (from ~19% to ~46%) during the first 42 days of adult life, despite there being no 16:1n-7 added to the diet. However, unlike the addition of 18:0 and 18:1n-9 to the diet, there were dramatic effects of additional dietary 18:2n-6 and 18:3n-3. For both of these PUFA, there were rapid and very large increases in their relative abundance in membrane lipids. For example, within the first week of adult life, the relative content of 18:2n-6 went from ~8% to ~25% of total membrane fatty acids when 18:2n-6 was added to the diet, while the respective increase in 18:3n-3 was from ~1% to ~28% when it was added to the diet. After 42 days the respective values were ~33% of membranes lipids for 18:2n-6 and ~35% for 18:3n-3. While there were dramatic increases in relative content of the 18-carbon PUFA, there was no change in 16:1n-7 content (unlike the other diets which all

resulted in a significant increase of 16:1n-7), and greater decreases in 16:0 and 18:1n-9, as well as in 20:4n-6 and 20:5n-3 than observed in blowflies on all other diets. The greater decreases in the two 20-carbon PUFA compared to blowflies fed the other diets is intriguing as it suggests the disappearance of the 20-carbon PUFA from membrane lipids is not compensated by their synthesis from their 18-carbon PUFA precursors and furthermore that the 18-carbon PUFA may be replacing some of the 20-carbon PUFA. The membrane lipids of blowflies fed SY diets either with no-added fat, or with added 18:0 and 18:1n-9, exhibited a decrease in PI throughout adult life (Supplementary figure 3). In experiment #3, the decrease in PI was identical in all of these three groups and was largely due to the decrease in the 20-carbon PUFA because of their substantial contribution to the calculation of PI. In blowflies fed the SY diet with the additional 18:2n-6, there was a lesser decrease in PI throughout adult life, while in those fed the diet with added 18:3n-3 there was only a very small decrease in PI. These PI differences between diet treatments were solely due to increased 18-carbon PUFA in membrane lipids.

Changes in membrane fatty acid composition measured in experiment #3 were not due to differences in the relative palatability of the food as there were no significant differences in rates of food consumption between the populations (see Figure 6). There also were no differences in maximum longevity (i.e. the average lifespan of the longest-living 10% of the population) between any of the diet treatments ($P = 0.72$), which implies no effect of these diets on ageing mechanisms (survival curves are presented in Supplementary Figure 4). Although there was no influence of diet on maximum longevity, the added 18:2n-6 resulted in a decreased average lifespan compared to the standard SY diet, while added 18:0, 18:1n-9, and 18:3n-3 all significantly increased average lifespan compared to the standard SY (no added fat) diet ($P < 0.0001$; see Figure 6).

Discussion

This contribution reports three experiments designed to experimentally test the 'membrane pacemaker' theory of ageing using the blowfly, *C. stygia*. This theory is based on extensive

observations of correlations between a species maximum lifespan and its membrane fatty acid composition, specifically the relative abundance of peroxidisable PUFA in membranes (for review see Hulbert et al. 2007). Without any knowledge of the regulation of membrane fatty acid composition in the blowfly, the first experiment sought to see if larval diets that differed in fatty acid composition would result in adult blowflies that differed in membrane composition and consequently different adult lifespans. This experiment showed that while the different larval diets affected aspects of the phospholipid fatty acid composition of emergent adults, they had no significant effect on adult longevity of the blowflies. Although the larval diets resulted in altered fatty acid composition of blowfly membrane lipids, many of the changes in peroxidisable fatty acids were compensatory, resulting in no significant effect of diet on the calculated peroxidation index (PI) of the membrane lipids. Despite dramatic differences in the PI of the different diets, the PI of blowfly membrane lipids remained relatively constant, implying they were 'homeostatically' regulated with respect to diet fatty acid profile (see Figure 2). This experiment was therefore unsuccessful as an attempt to test the 'membrane pacemaker' theory of ageing as we were unable to experimentally alter the PI of blowfly membrane lipids by the different larval diets. This outcome, however, did increase our understanding of the regulation of membrane composition in this insect. Such a lack of dietary effect on membrane PI is similar to the situation recently observed for the laboratory rat, where an extensive study comparing the effect of twelve moderate-fat diets (identical except for their fatty acid profile) showed that SFA, MUFA and PUFA content of membrane lipids is 'homeostatically' regulated (Abbott et al., 2012) and PI of membrane lipids is also constant despite wide variation in diet PI (Hulbert et al., 2013).

Following this unsuccessful attempt to test the membrane pacemaker theory by modification of larval diet, a further experiment (expt #2) was carried out to examine if modification of the fatty acid profile of the adult blowfly diet could alter adult longevity. To this end, the standard sugar-yeast-agar (SY) diet, commonly used for maintaining laboratory colonies of *Drosophila*, was modified by adding different vegetable oils, in order to provide diets with different fatty acid

profiles. We have previously shown that too much olive oil added to this SY diet can have detrimental effects on adult lifespan of this blowfly (Ujvari et al., 2009). Low-fat diets (either 1-3 g/100 ml of diet) either significantly increased or had no effect on maximal adult longevity whereas high-fat diets (5-15 g/100 ml) significantly decreased blowfly adult longevity. Furthermore, we demonstrated this dramatic effect of a high-fat diet on blowfly adult lifespan was not associated with alterations in membrane fatty acid composition of blowflies measured at both day 0 and day 10 post-eclosion (Ujvari et al., 2009). In the second experiment reported here, examining the influence of fatty acid profile of adult diet on longevity, all experimental diets were of low-fat composition (3 g/100 ml).

As well, the adult blowflies used in this second experiment were obtained as pupae from a commercial breeder of *C. stygia* and randomly divided among the different diet treatments. This commercial breeder maintains a huge out-breeding colony of this blowfly to provide blowfly larvae as 'bait' for fishers throughout Australia and also provide adult blowflies as "glasshouse pollinators" for agricultural firms. The food provided for this commercial blowfly population is waste from a fish abattoir and thus this was the larval food of the adults used in this part of the study. To follow the influence of the different adult diets we measured fatty acid composition of whole blowfly phospholipids at days 0, 21 and 42 post-eclosion.

The commercial purchase of blowfly pupae for experiment #2 meant the total number of adult blowflies used in this experiment (=2052 blowflies) was an order of magnitude greater than that used in the first experiment (n=133 in expt #1a; n=280 blowflies in expt #1b) and consequently gave greater power in determination of any diet effects on lifespan. This second experiment showed that, although manipulation of the fat profile of the adult diet had statistically significant effects on membrane fatty acid composition, there was no significant difference between dietary treatments in PI of blowfly phospholipids after 42 days on the diet. Diet did affect both average and maximal adult lifespan, these effects although statistically significant were very small and in the opposite direction to the membrane pacemaker theory prediction (see Figure 4). In this experiment the

changes in membrane fatty acid composition of blowfly phospholipids observed were overwhelmed by age-related changes that were little influenced by adult diet treatments.

During the adult life of the blowflies in experiment #2, there was a decrease in both SFA and PUFA content and an increase in MUFA content of their phospholipids (see Table 2). At the beginning of their adult life (i.e. day 0 post-eclosion), there were significant amounts of 20- and 22-carbon PUFA that were the dominant (>80%) contributors to the calculated PI value. By day 42 of adult life the relative abundance of these highly polyunsaturated membrane fatty acids had decreased by approximately two-thirds and this decrease was major reason why the PI value of their phospholipids approximately halved during time. Essentially the only effect of vegetable oil-supplemented adults diets on membrane composition was that the two PUFA-rich vegetable oils (i.e. safflower oil and flaxseed oil) resulted in an enhanced relative abundance of their dominant fatty acids (respectively 18:2n-6 and 18:3n-3), while there was no effect of either the SFA-rich or MUFA-rich vegetable oils on membrane fatty acid composition.

Vegetable oils often contain other fat-soluble molecules, notably antioxidants, which are natural components of the seeds from which they are obtained. Often the higher the PUFA content of these oils, the higher the content of antioxidants responsible for scavenging oxidising free radicals. For example in a comparative study of the total free radical scavenging capacity (TSC) of various vegetable oils, linseed (= flaxseed) oil had TSC of 81.1 units, while safflower oil TSC was 71.7 units and olive oil had a TSC of 27.2 units (Espin et al., 2000). While this comparison did not provide a TSC value for coconut oil, being a SFA-rich oil it will have low antioxidant content. For example, the USDA food composition website (http://ndb.nal.usda.gov/) reports the vitamin E content of coconut oil to be 0.09 mg/100 g compared to 14.35 mg/100 g for olive oil and 34.10 mg/100 g for safflower oil. The presence of natural antioxidants in vegetable oils, especially in the PUFA-rich oils may have been responsible for the small increases in adult lifespan of blowflies in experiment #2. The third experiment was performed in the same manner as experiment #2, except that adult diets were enriched with individual fatty acid as pure methyl esters (and thus had no

additional vegetable oil based antioxidants) and suggests this may have been the case. In experiment #3 there was no significant effect of adult diet fatty acid profile on maximum lifespan of blowflies. While additional diet 18:0, 18:1n-9, 18:3n-3 all had a small beneficial effect on average lifespan of blowflies compared to no added diet fatty acids, there were no significant differences between the SFA, MUFA or n-3 PUFA enriched diets. When pure 18:2n-6 was the added diet fatty acid, blowflies had significantly shorter average adult lifespan (although no change in maximum lifespan) of blowflies. This was the opposite effect of the additional dietary 18:2n-6 provided as safflower oil in experiment #2 and in experiment #3 was largely due to a very early die-off of some blowflies.

Assessing membrane fatty acid composition of phospholipids extracted from whole blowflies measures the fatty acid composition of all cellular membranes from all tissues combined. It is possible that such measurements of combined membrane lipids may mask significant diet-induced changes in more specific subcellular membranes proposed to be important in the processes of ageing and lifespan determination, namely mitochondrial membranes. The observation from experiment #3 that changes in the fatty acid composition of total blowfly phospholipids were identical to those recorded for blowfly thorax mitochondrial phospholipids shows this is not the case.

There was no information available regarding the mechanisms regulating membrane fatty acid composition of blowflies when we commenced these studies assuming it would be relatively easy to modify membrane composition by diet manipulation. We have found this is not the case and while we have not been able to definitively test the membrane pacemaker theory of ageing, we have gained insight into some of the mechanisms involved in determining membrane fatty acid composition of the blowfly (and likely other insects) and how this differs from the situation in mammals. Three aspects of the processes involved in regulation of membrane fatty acid composition in blowflies are worthy of brief comment. These are: (i) whether PUFA are essential components of the diet; (ii) the enzymatic conversion of dietary fatty acids to more unsaturated and

longer-chain fatty acids; and (iii) the selective incorporation of fatty acids into membrane phospholipids.

In mammals, SFA and MUFA are not essential in the diet because they can both be synthesised from non-lipid sources. However, both n-6 PUFA and n-3 PUFA are essential components of the diet because they cannot be synthesised *de novo* due to a lack of the desaturase enzymes required. In lower animals, such as *C. elegans*, no fatty acids are essential in the diet, as they have a wide range of desaturase and elongase enzymes and can synthesise all types of fatty acids *de novo* from non-lipids (see Hulbert et al., 2013). Although some insects have been shown to be capable of *de novo* synthesis of 18:2n-6 (but not 18:3n-3), this is not the case for *Drosophila* and it is likely that for all Diptera (including blowflies) both 18:2n-6 and 18:3n-3 are essential dietary fats (Cripps et al., 1986). Avid uptake of 18:2n-6 and its incorporation into lipids of *Drosophila* has been previously reported (Draper et al., 2000). In all three of our experiments, membrane lipids of newly-eclosed blowflies contained both 18:2n-6 and 18:3n-3 and these were likely obtained preformed from the larval diet. When these PUFA were provided in the adult diet, they were avidly incorporated into membrane lipids but, if they were unavailable in the adult diet, there was a continual gradual decrease in their relative abundance in membrane lipids throughout adult life of the blowfly, which is consistent with their inability to synthesise them *de novo*.

In mammals, dietary 18-carbon PUFA are converted to 20-carbon PUFA by Δ5-desaturase and Δ6-desaturase and elongase enzymes. In all our blowfly experiments, the membrane lipids of newly-eclosed blowflies contained significant amounts of 20-carbon PUFA that were likely sourced from their larval diet. There were no 20-carbon PUFA in any of the adult diets used in this study and there was a substantial decline in the 20-carbon PUFA content of blowfly in every treatment group, despite the fact that some treatment groups showed dramatic increases in the relative abundance of their 18-carbon PUFA precursors in the blowfly membrane lipids (see Figure 5). This likely indicates the inability of blowflies to synthesise 20-carbon PUFA. This may be true for all Diptera, as genes for seven different Δ9-desaturase enzymes (responsible for production of 16-

carbon and 18-carbon PUFA from SFA) but no genes for Δ5-desaturases or Δ6-desaturases have been identified in the *Drosophila* genome (Fang et al., 2009). Thus it is understandable that only 18-carbon PUFA are reported for phospholipids of *Drosophila* fed normal SY food (Hammad et al., 2011; Moghadam et al., 2013) as these will be likely obtained preformed from the yeast in the food, which generally do not contain 20-carbon PUFA (Hulbert, unpublished observations).

Interestingly, in these blowflies, the presence of 16:1n-7 in membranes increased over time often independently of diet conditions. This indicates that factors other than dietary fatty acids influence membrane fatty acid composition, since 16:0 can be synthesised from non-lipid sources and consequently desaturated to 16:1n-7 by the action of the Δ9-desaturase enzyme. While this increase was not related to changes in longevity in the current study, an increase in 16:1n-7 has previously been shown in the offspring of nonagenarians compared to age-matched controls (Puca et al., 2008) as well as in other situations (Cohen et al., 2011; Ghezzo et al., 2013). In mammals, the fatty acid composition of membrane lipids is determined by the combined actions of *de novo* synthesis of specific molecular species of phospholipids and their remodelling via deacylation/reacylation processes (see Hulbert et al., 2013). These processes can be specific for individual fatty acids and this also appears to be the case for blowfly membrane lipids. For example, in experiment #3 there was dramatic incorporation of both 18:2n-6 and 18:3n-3 into membrane lipids, but no increased incorporation of 18:0 and very limited incorporation of 18:1n-9 despite the fact that all these fatty acids were provided at the same concentration in the different foods. There were similar differences in relative incorporation of these fatty acids in experiment #2 where adult diets were supplemented with vegetable oils. Another example of selective incorporation into membrane lipids is the observation that 22:6n-3 was a substantial component of the larval diet for all blowflies but absent in blowfly phospholipids. The processes involved in determining fatty acid composition of blowfly phospholipids likely differ between the larval and adult life stage. Although 20:5n-3 was incorporated into blowfly membrane lipids when available in the larval food, we were unable to increase its incorporation into blowfly phospholipids when we

supplemented the adult SY diet with it either in the form of fish oil or krill oil (Kelly and Hulbert, unpublished results). Thus in the adult blowfly, highly polyunsaturated fatty acids (such as 20:5n-3 and 22:6n-3) are not incorporated into membrane lipids even when available in excess, and in this respect, regulation of membrane composition in this insect is very different to mammals. Due to this low level of membrane unsaturation, these organisms may be poor models for the examination of mammalian ageing in relation to membrane fatty acid composition. This is especially relevant when considering the large influence these highly polyunsaturated fatty acids have on age-related disease such as inflammation and cardiovascular disease in mammals (see Stables and Gilroy, 2011; Jump et al., 2012; Gerber et al., 2013). The "membrane pacemaker theory" of ageing emerged from measurements of the membrane fatty acid composition of mammals and the inverse correlation between the PI calculated for these membrane fatty acid composition data and the species maximum lifespan (e.g. Pamplona et al., 1998; Hulbert, 2005). It was also supported by observations that; (i) birds, which are generally much longer-living than similar-sized mammals, have membrane lipids with a low PI values and appear to follow the same relationship as mammals (Hulbert et al., 2007), and (ii) that exceptionally long-living mammals (Mitchell et al., 2007; Hulbert et al., 2008), and birds (Buttemer et al., 2008) have membrane lipids with relatively low PI values. However, it is not a universal observation among birds, as a recent comparison of long-living parrots and short-living quail showed the parrots to have membrane lipid PI commensurate with their maximum lifespan but that quail had the same PI values as the parrots and thus much lower values than one might expect for their maximum longevities (Montgomery et al., 2012) thus, factors other than membrane fatty acid composition are likely responsible for the relatively short lifespan of quail.

Although, in general, birds and mammals appear to share the same lifespan-PI relationship, it is obvious that invertebrates do not obey this equation. For example, if blowflies obeyed the mammal-bird relationship, from their PI values we would expect the adult lifespan of blowflies to be measured in years not days! However, there are invertebrate examples of connections between

membrane composition and longevity. Long-living bivalve molluscs have membrane lipid composition with a lower PI than shorter-living bivalves (Munro and Blier, 2012). Long-living mutant *C. elegans* also have membrane fatty acids with a low PI compared to normal-living controls (Shmookler Reis et al., 2011). Although they have the same genome, long-living queen honeybees have membrane lipids with a lower PI than the shorter-living worker honey-bees (Haddad et al., 2007). In *Drosophila melanogaster*, flight activity has been shown to shorten lifespan and also increase the PI of their membrane lipids (Magwere et al., 2006), however, it has also been recently reported that there were no differences in the fatty acid composition of phospholipids long-living strains of *D. melanogaster* compared to shorter-living controls (Moghadam et al., 2013).

In the experiments reported here we were unable to consistently and substantially alter the adult lifespan of the blowfly *C. stygia* by relatively extreme manipulations of diet fat profile. We have concluded that this was largely because although diet manipulation does alter the relative abundance of some individual fatty acids, that membrane PI is, in general, refractory to considerable diet-induced changes. This was true regardless of whether alterations were made to the larval or adult diets. Other means of experimentally altering membrane fatty acid composition may be more successful. For example, dietary supplementation with exogenous 20:5n-3 and 22:6n-3 in *C. elegans* did not change their longevity and also did not change the total PUFA content of their phospholipids (Hillyard and German, 2009). In contrast, use of dietary RNAi against the fat-4 desaturase enzyme both extends lifespan and increases resistance to oxidative stress in *C. elegans* (Shmookler Reis et al., 2011) and although theses authors did not measure whether membrane fatty acid composition was changed in these experiments, others (Watts and Browse, 2002) report the fatty acid composition of fat-4 mutant compared to wild-type *C. elegans* for which we have calculated PI values of 123 versus 174 respectively (see Hulbert et al., 2013). Use of non-dietary techniques, such as RNAi, to alter membrane fatty acid composition may be more productive in experimentally testing the membrane pacemaker theory of ageing.

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Figure legends.

Figure 1. Fatty acid composition of (i) total lipids of larval food (ii) phospholipids of whole larvae, and (iii) thoracic phospholipids of adults fed these diets as larvae. The six different types of larval food are identified on the left-hand side of the figure (see methods for a full description). Each pie chart shows the relative composition of fatty acids (as percent of total fatty acids) in the following clockwise order: saturated fatty acids (grey); monounsaturated fatty acids (diagonal stripes); n-6 polyunsaturated fatty acids (white); n-3 polyunsaturated fatty acids (stippled). Where pie sectors are large enough, individual fatty acids are identified by numbers representing "number of carbons: number of double bonds". In all pie charts, individual fatty acids are presented in the following clockwise order: 16:0, 18:0, other saturates, 16:1n-7, 18:1n-9, other monounsaturates, 18:2n-6, 20:4n-6, 18:3n-3, 20:5n-3, 22:6n-3.

Figure 2. Relationship between the Peroxidation Index (PI) of phospholipids (larval and adult thorax) and PI of the larval diet, in *Calliphora stygia*. Dotted line represents the line of perfect conformity between diet and blowfly lipids. Data is presented as means ± S.E.M, where error bars are not obvious, error is less than the size of the marker.

Figure 3. Average and maximum lifespan of adult *Calliphora stygia* fed different diets during their larval development. See text for details of diets. For both average and maximum lifespan, error bars when shown are ± S.E.M. In experiment #1a maximum lifespan is average of three longest-living blowflies. In all other situations sample size is identified within each bar. There were no significant differences between treatments for either average or maximum lifespan in both expt #1a and #1b.

Figure 4. Average and maximum lifespan of adult *Calliphora stygia* fed diets supplemented with vegetable oils during their adult life. See text for details of diets. For both average and maximum lifespan, error bars are ± S.E.M. Maximum lifespan determined as the average of the longest-lived 10% of the population. Within both average and maximum lifespan, treatments with different letters are significantly different ($P<0.05$).

Figure 5. Changes in the relative content of the main fatty acids in phospholipids of whole blowfly (left hand graphs) and in isolated thorax mitochondria (right hand graphs) during adult life of *Calliphora stygia*. * represents a significant difference between diets at that day (* P

<0.01, ** P <0.001, ***P <0.0001). Values are shown as means ± SEM (N = 4, except for day 0 mitochondria where N = 3). If no SEM bar is evident, it is smaller than the symbol.

Figure 6. A) Daily food consumption per blowfly and B) average and maximum lifespan of adult *Calliphora stygia* fed either SY diet or SY diets enriched with individual 18-carbon fatty acid methyl esters during their adult life. Values are mean ± SEM and N= is shown inside each food consumption bar in graph B. Maximum lifespan determined as the average of the longest-lived 10% of the population. There were no significant differences between diets in both food consumption and maximum lifespan. For average lifespan treatments with different letters are significantly different (P<0.05).